Regulation of inositol 1,4,5-trisphosphate receptor expression

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Chapter 2

Receptor regulation: an overview

2.1 Introduction:

Receptors are the eyes and ears of the cell. Even the simplest of life forms possess receptors, enabling them to respond to changes in chemical concentration, oxygen levels, pH, the intensity or wavelength of light, temperature and in some cases even the Earth's magnetic field (Armitage, 1992).

In multicellular organisms, intercellular communication is also mediated through receptors, e.g. for hormones, growth factors, morphogens and neurotransmitters.

Besides their function in detecting changes in the cell's environment, receptors are also important for intracellular communication, e.g. the intracellular receptor for inositol 1,4,5-trisphosphate plays a central role in this thesis.

Although the genetic content is identical for all somatic cells, not every cell-type expresses the same set of receptors. In addition, the expression of a given receptor within one cell-type fluctuates over time during embryogenesis, homeostasis or disease. Therefore, studying receptor expression and regulation is of paramount importance in understanding biological systems and disease.

Receptor regulation can be achieved through changes in its synthesis, changes in its properties and by changes in its rate of degradation (Fig. 1.). In this chapter, an overview will be given of these three basic mechanisms of receptor regulation. Although similarities exist with prokaryotic receptor regulation, only eukaryotic receptor regulation will be discussed.

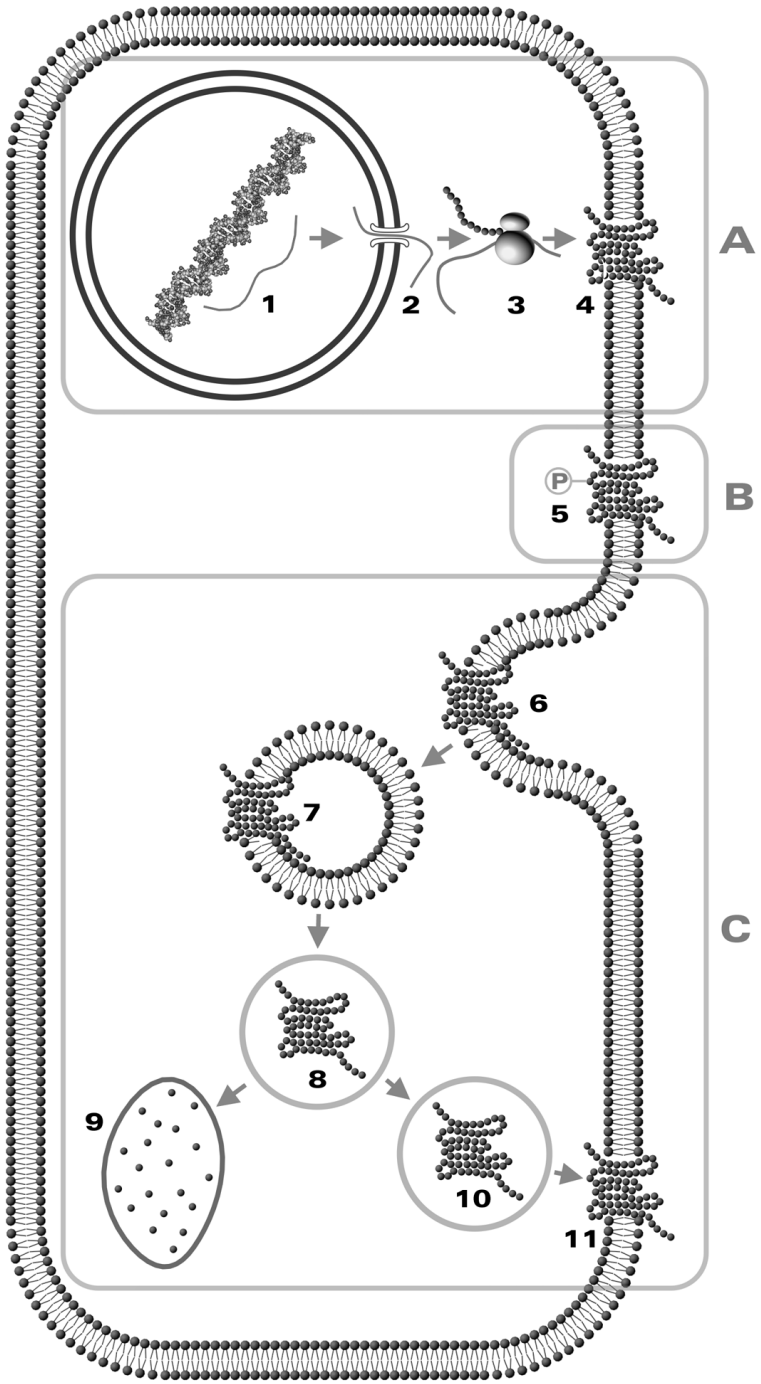

Fig. 1 Life of a receptor A) Receptor levels can be regulated through changes in the rate of synthesis. 1) The information stored in the receptor gene is transcribed onto a mRNA molecule. 2) The mRNA molecule is subsequently exported from the nucleus to the ribosomes. 3) In a process called translation, the information stored on the mRNA molecule is translated into a polypeptide. 4) The polypeptide is processed into a mature receptor. **B)** Receptor function can be regulated by changing its properties. 5) The receptor can be desensitized by phosphorylation. **C)** Receptor levels can be regulated through changes in its rate of degradation. 6) The plasma membrane receptor gets internalized. 7-8) The internalized fragment of plasma membrane containing the receptor is transported to an endosome. In the process of endosomal sorting, the receptor may be directed to a lysosome for degradation (9) or it may be recycled (10-11). En, endosome; Ly, lysosome; Np, nuclear pore; Nu, nucleus; Pm, plasma membrane; Rb, ribosome.

2.2 Receptor synthesis

To understand the mechanisms of receptor regulation, it is important to understand the basic mechanisms of protein synthesis. In short, the aminoacid sequence of proteins is encoded on the DNA in the form of genes. These genes are grouped together in complex structures called chromosomes, which reside within the nucleus of the cell. In a process called transcription, the genetic information of the gene is copied onto a messenger RNA (mRNA) molecule. The mRNA molecule is then transported from the nucleus to the ribosomes, which reside in the cytosol. In a process called translation, the genetic information on the mRNA molecule is translated into a polypeptide chain. This polypeptide may then be processed, resulting in a mature protein or receptor.

2.2.1 Chromatin structure

The building plans of proteins, including receptors, are encoded by genes, which reside on chromosomes. These chromosomes are made of chromatin, a complex structure of DNA molecules and proteins. Chromatin has a beadlike appearance due to the presence of nucleosomes, regular complex structures of histone proteins and DNA.

Early electron microscope studies already showed that alterations in the chromatin structure is associated with active genes (Hill, 1979; Trendelenburg, 1983; Gottesfeld and Butler, 1977). Therefore, the first step in receptor regulation is controlled by the accessibility of the chromatin for the factors involved in transcription.

Highly complexed or condensed chromatin does not seem to allow efficient gene transcription, whereas open chromatin or euchromatin is associated with active gene transcription. The less complexed structure of euchromatin also makes the DNA in the chromatin susceptible to the action of deoxyribonuclease I (DNAse I). This property of euchromatin has enabled the exact mapping of structural changes within the chromatin.

Structural chromatin changes at the time of transcription have been reported for a number of genes, including the genes that code for the acetylcholine receptor. E.g. during myogenesis, the transcription of the different subunits of the acetylcholine receptor was associated with structural changes of the corresponding chromatin (Crowder and Merlie, 1988).

Several signaling systems have been implicated in the mechanisms leading to changes in chromatin structure, including the steroid/ nuclear receptor signaling pathway. These steroid receptors interact directly with proteins in the

condensed chromatin and are able to recruit the necessary activities for chromatin remodeling (Hager et al., 1998)
The main function of the transition from condensed chromatin to euchromatin is to enable the binding of proteins involved in transcription to the DNA (Rusterholz et al., 1999). Although DNA is less condensed in euchromatin, nucleosomes may play an important role in transcriptional regulation as nucleosomes seem to facilitate the binding of transcription factors to the DNA at specific sites of the gene (Ioshikhes et al., 1999).

2.2.2 Gene transcription: from DNA to mRNA

Once the gene within the chromatin is accessible, transcription may commence. Basically, a gene consists of two elements, the coding sequence (CDS) and regulatory domains. The coding sequence codes for the aminoacid sequence of the protein, whereas the regulatory domains control the transcriptional rate of the gene. The regulatory domains that control transcription can be divided into three elements: the promoter, repressors and enhancers.

2.2.3 The promoter

The promoter is the site where RNA polymerase binds to the gene and is usually situated directly upstream of the CDS. The majority of eukaryotic peptide encoding genes are transcribed by the enzyme RNA polymerase II and therefore only the regulation of these genes will be discussed.
The function of the promoter is to faciltate the formation of the transcription initiation complex, a complex structure of RNA polymerase II and several transcription factors. Eukaryotic promoters exhibit a complex architecture and usually consist of a number of distinct promoter elements. These elements are specific nucleotide sequences or motifs that can bind specific transcription factors. Some elements are commonly found in promoters like the so-called TATA-box, the cap signal, the CCAAT-box and the GC-box (Bucher, 1990)(Table 1).

Table 1.
Promoter elements that are commonly found in eukaryotic promoters.

Promoter element:	Nucleotide sequence*:	Transcription factor:
TATA box	TATAWAW	TFIID, TBP and TAFs
Cap signal	CANYYY	-
CCAAT box	CCAAT	CBF, NF-Y, CP-1 and alpha-CP1
GC box	GGGCGG	Sp-1

* Nucleotide sequence in IUPAC code

The TATA-box is found in the majority of promoters and is characterized in part by the nucleotide sequence TATA (Xu et al., 1991). An early study on the TATA-box showed that the transcription factor TFIID binds to the TATA-box (Matsui et al., 1980).

TFIID is a multiprotein complex consisting of TATA-binding protein (TBP, for review see (Goodrich and Tjian, 1994)) and TBP-associated factors (TAFs, for review see (Verrijzer and Tjian, 1996)). Binding of TBP to the TATA-box induces dramatic distortion of the DNA helix (Kim et al., 1993; Kim et al., 1993) and therefore TFIID seems to be essential for the formation of the preinitiation complex and the recruitment of RNA polymerase II. However, several studies have demonstrated that TFIID is also involved in regulating TATA-less promoters (Pugh and Tjian, 1991; Wiley et al., 1992; Yoganathan et al., 1993; Yoganathan et al., 1992). It seems that TAFs but not TBP are required for transcription initiation of these TATA-less promoters (Martinez et al., 1994).

The cap signal, a CA motif followed by several cytosine and/ or thymidine nucleotides, is present in about 60% of all promoters. Although the cap signal is usually found near the initiation site, its function is still obscure.

The CCAAT and GC box are elements that are active in either orientation and are found at variable distances from the initiation site.

The CCAAT box is named after its nucleotide motif and is recognized by the transcription factors CBF, NF-Y, CP-1 and alpha-CP1. The involvement of the CCAAT box in receptor regulation has been demonstrated for a number of receptors, including the type II transforming growth factor β receptor (Kelly et al., 1998), the platelet-derived-growth-factor β receptor (Takata et al., 1999; Kitami et al., 1998; Ishisaki et al., 1997) and the very low density lipoprotein receptor (Kreuter et al., 1999).

The GC-box is a G rich motif and is recognized by the ubiquitously expressed transcription factor Sp-1. The involvement of Sp-1 in gene transcription has been demonstrated for many receptor genes, e.g. the type II TNF receptor (Bethea et al., 1997).

Although TATA-box, the cap signal, the CCAAT-box and the GC-box can be found in many genes, specific receptor regulation can be achieved through these generalized elements as demonstrated by the cAMP-induced transcription of the progesterone receptor through the GC- and CCAAT-box (Park-Sarge and Sarge, 1995).

Besides these generalized transcription factors, many other transcription factors are involved in regulating gene expression. Although the overall transcriptional

regulation is the result of the complex interaction of many transcription factors, the function of a number of transcription factors seems more specific.
An example of such transcription factors is the cAMP-response-element-binding protein (CREB). Increased levels of the second messenger cAMP activates cAMP-dependent protein kinase (protein kinase A). Subsequently, protein kinase A activates CREB by phosphorylation (Gonzalez et al., 1989). Activated CREB then binds as a homodimer to the motif TGACGTCA, known as the cAMP-response-element (CRE). In addition, several variants of the CRE have been described (Benbrook and Jones, 1994). Upon binding, the CRE/CREB complex is bridged to the basal transcriptional apparatus by the co-activator protein CREB-binding-protein (Andrisani, 1999), contributing to activation of the promoter.
The involvement of CREB in regulating transcription has been demonstrated for many genes, including the rat adrenergic receptor gene (Yang et al., 1997).
Although the characterization of promoters can be a time-consuming task, the isolation and cloning of promoters gave researchers the tools to easily monitor gene expression through the use of promoter reporter constructs. Using recombinant DNA techniques, the gene of an easily measurable protein (called reporters) can be placed under control of the promoter of interest. When reintroduced into cells, these constructs enable easy monitoring of gene expression. Commonly used reporters are chloramphenicol acetyltranferase, β-galactosidase, firefly luciferase and green fluorescent protein. The latter even allowed monitoring of gene expression in living organisms and has greatly facilitated the study of the genes that are involved in development.
Although reporter gene constructs allow easy monitoring of gene expression, care should be taken in interpreting the results of such experiments, as the regulation of isolated promoters may differ from the endogenous promoter. These differences may arise from several causes. First, the isolated promoter may not be complete and may therefore lack regulatory upstream elements. Secondly, the isolated promoter may no longer be under the influence of an enhancer (see chapter 2.2.5). Finally, differences in the chromatin structure of the reintroduced promoter and the endogenous promoter may cause differences in gene regulation.

2.2.4 Repressors or silencers

Repressors or silencers are negative regulators of transcription. They are so called trans-acting control elements, meaning that they are not confined in their

binding to the DNA that carries their genetic information. Repressors usually bind within the promoter region and thus block binding of RNA polymerase.
A well-studied repressor is the Neuron-restrictive Silencer factor/RE1-silencing transcription factor (NRSF/REST). NRSF/REST is thought to be a master negative regulator of neurogenesis, although recent studies suggested that NRSF/REST also has a role in neuronal activity-implied processes (Palm et al., 1998). The element that binds NRSF/REST has been found in the promoter of a number of neuronal genes, including the rat m4 muscarinic acetylcholine receptor gene (Mieda et al., 1997). Upon binding, NRSF/REST does not simply block the binding of RNA polymerase, but appears to exert its silencing effect through the interaction with positive transcription factors (Mieda et al., 1997).

2.2.5 Enhancers

Enhancers are positive regulatory elements of transcription and can be situated far from the promoter, either downstream or upstream of the promoter. The motifs of enhancers are usually larger than those of transcription factors and can be active in either orientation.
An example of a classic powerful enhancer has been described in the human bradykinin B_1 (BKB_1) receptor gene (Yang et al., 1998), in which a 100 bp motif strongly enhanced gene transcription. Further, the cell-type specific expression of the human BKB_1 receptor gene seemed to be controlled by this enhancer.

2.2.6 From primary RNA to mRNA.

Transcription will start when the transcription initiation complex, consisting of RNA polymerase II, TFIID and many other transcription factors, is complete. During transcription, the genetic information stored in the gene is copied onto a primary RNA transcript. This primary transcript is then converted to a mature mRNA molecule by removing the introns (see chapter 2.2.7) and by the addition of a poly(A) tail and a 7-methylguanosine cap structure.

2.2.7 Intron-exon structure

The intron-exon structure of a gene allows the synthesis of receptor peptides with alternative aminoacid sequences.
Within a gene, the CDS is usually situated directly downstream of the promoter. The CDS codes for the aminoacid sequence of the protein and those parts of the gene that contain CDS are called exons. The CDS does not need to be continuous but can be interrupted by noncoding introns. The number of

exons can vary extensively. For example, the CDS of the gene encoding the human angiotensin II type 1 receptors is situated on 1 exon (Furuta et al., 1992), whereas the human ryanodine receptor is encoded by 106 exons (Phillips et al., 1996).

During transcription, the DNA sequence of the introns and exons is copied onto a primary RNA transcript (Fig. 2.). Then, in a poorly understood process called splicing, the exons within the primary RNA transcript of a gene are fused together.

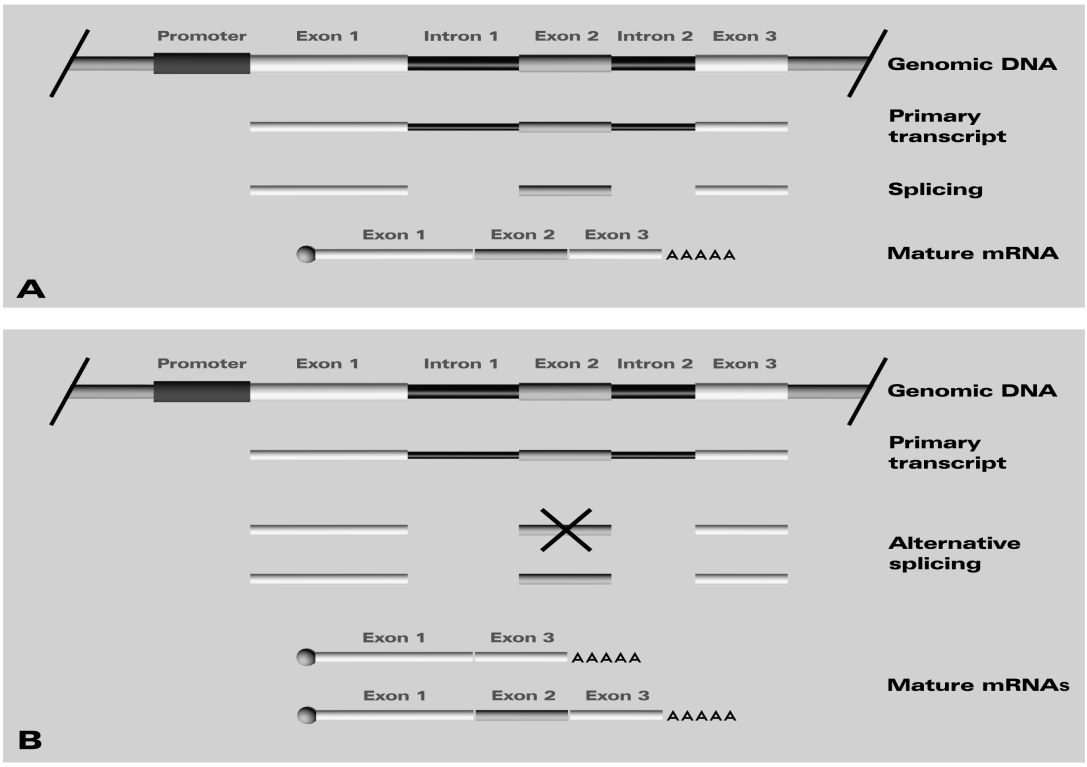

Fig. 2 Intron-exon splicing. The peptide sequence of proteins is stored within genes on exons. These exons are interrupted by noncoding regions called introns. During transcription, both the intron and exon sequences are copied onto a primary transcript. **A)** In a process called splicing, the exons are joined together, resulting in a mature mRNA. **B)** Dependent on cell type and developmental phase, the primary transcript may be alternatively spliced, allowing the synthesis of different receptor proteins from one gene.

Two types of splicing processes have been described: constitutive splicing and alternative splicing. In the process of constitutive splicing (Fig. 2A), the fusion of exons is mainly determined by consensus donor and acceptor splice sequences. In alternative splicing (Fig. 2B), the rearrangement of exons is dependent on cell type and developmental phase, allowing the synthesis of different proteins from one gene. For example, the 1,4,5-inositol trisphosphate receptor type I contains two alternative splice sites, SI and SII (Mignery et al., 1990; Nakagawa et al., 1991). Although the exact function of these splice

forms are not fully understood, the alternative splicing of SI and SII seems to be neuron specific (Schell et al., 1993). Further, the presence of introns within a gene seems to increase the rate of transcription (Brinster et al., 1988).
Besides enabling the formation of different proteins from one gene, the exon-intron structure of the primary RNA transcript may also influence mRNA export processes as has been demonstrated in C. elegans (MacMorris et al., 1999).
Although the noncoding introns are removed, the mature mRNA usually still contains two regions that do not get transcribed or translated. These regions are the so-called 5' and 3' untranslated regions (UTR) and they play an important role in regulating mRNA stability (see chapter 2.2.8) and translation (see chapter 2.2.10).

2.2.8 mRNA stability

The rate of receptor synthesis is also influenced by the half-life or stability of the mRNA. The majority of mature mRNAs possess a 7-methylguanosine cap structure and a 3'poly(A) tail of up to 200 adenosine residues in length. Both the cap and the poly(A) tail are stabilizing elements, protecting the mRNA from the action of exonucleases.
The stability of mRNA molecules can be influenced through three mechanisms. The first major pathway involves shortening of the poly(A) tail or deadenylation. After deadenylation, the cap is removed, leaving the mRNA susceptible to the action of 5'-3' exonucleases. Alternatively, the mRNA can be destabilized through deadenylation and subsequent 3'-5' exonuclease degradation. Degradation of specific mRNAs can be mediated through specific sequence elements (AU-rich elements) on the mRNA by enhancing rapid deadenylation. For example, the stability of the human B_2-adrenergic receptor mRNA is mediated through specific AU-rich elements (Danner et al., 1998).
The second pathway involves deadenylation-independent decapping of the mRNA and subsequent 5'-3' exonuclease degradation. This mechanism appears primarily involved in the degradation of incorrectly processed intron containing mRNAs (Muhlrad and Parker, 1994).
Finally, mRNA can also be degraded by endonucleolytic cleavage of the mRNA by specific endonucleases. For example, endonuclease cleavage sites have been identified in the 3' untranslated region of the mRNA of the human transferrin receptor (Binder et al., 1994).

2.2.9 *Nuclear export*

Once a mature mRNA is formed, it is exported from the nucleus and transported to the ribosomes. Although specific signals in the mRNA may influence these processes (MacMorris et al., 1999), the significance of nuclear export pathways has not been explored to its full capacity yet..

2.2.10 Translation

The rate of receptor synthesis may also be controlled by the rate of translation. During translation, the CDS of the mRNA is translated into a peptide and the efficiency of this process depends largely on the structure of the 5' UTR of the mRNA. For example, the expression of the human α folate receptor is in part determined by the composition and length of its 5' UTR (Roberts et al., 1997). The translation process is characterized by three stages: initiation, elongation and termination (Fig. 3.).

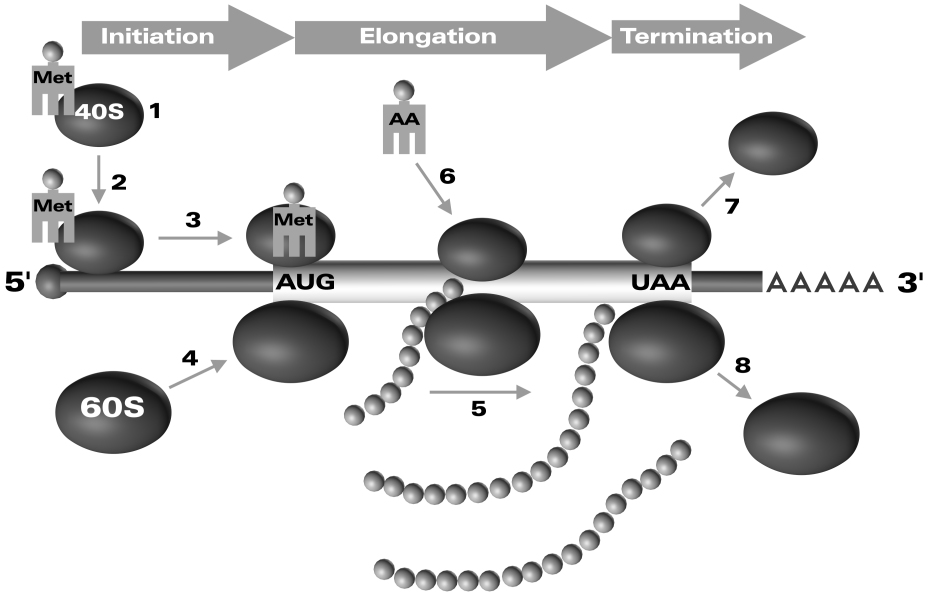

Fig. 3 Translation. In the process of translation, the information stored on the mRNA molecule is translated into a polypeptide. This process is characterized by three stages: initiation, elongation and termination. The rate of protein and receptor translation may be controlled through regulation of each of these stages. (1-4) Initiation: 1) the first step in initiation is the binding of the first transfer RNA (Met-RNA) to the small ribosomal subunit, a process that is under control of initiation factor eIF2. 2) Upon formation, this complex binds to the cap-binding factor eIF4E, which facilitates the binding to the 7-methylguanosine cap of the mRNA. 3) The initiation complex moves towards the initiation codon. 4) Subsequent binding of the large ribosomal subunits results in a active translation complex. (5-6) Elongation: the rate of protein (and receptor) synthesis is controlled by the rate of elongation. In most eukaryotes, the rate of elongation is controlled by the elongation factors eEF-1 and eEF-2. (7-8) Termination: translation is terminated by the two eukaryotic release factors eRF1 and eRF3.

The initiation factors eIF2 and eIF4E play crucial roles in translational control. The main function of eIF2 is to form a complex with the first transfer RNA (Met-tRNA) and the 40S ribosome. Upon formation, this complex binds to the cap-binding factor eIF4E, which facilitates the binding to the 7-methylguanosine cap of the mRNA. When transcription is complete, eIF2 is recycled from its inactive form eIF2:GDP to the active form eIF2:GTP by the factor eIF2B. This initiation step can be regulated at different levels. Active eIF2:GTP can be phosphorylated in response to several stress conditions or by release of calcium from intracellular stores (Reilly et al., 1998). Phosphorylation of eIF2:GTP results in a higher affinity for eIF2B, leaving less eIF2B available for recycling inactive eIF2:GDP. This mechanism leads to a generalized down-regulation of protein synthesis. Further, eIF2B itself may be regulated through phosphorylation.

The function of eIF4E may be regulated through eIF4E levels, through phosphorylation of eIF4E and by specific inhibitors of eIF4E.

Regulation of the translation initiation factors mainly controls generalized protein synthesis rather than that of specific transcripts. However, specific regulation of individual transcripts may occur if these transcripts differ in their dependence on eIF4E. This has been demonstrated for heat shock proteins (Joshi-Barve et al., 1992), but to date it is unclear whether the same mechanism is employed in the regulation of receptors.

Translational initiation may also be regulated through structural elements in the UTRs of the mRNA molecule. RNA molecules may form secondary structures known as hairpin-loops or stem-loops. The interaction of RNA binding-proteins with these RNA loops may effectively block the binding or function of the translation initiation complex. A classic example of this mechanism is the regulation of the transferrin receptor by iron response elements or IREs (Haile et al., 1989). IREs are specific stem-loop structures in the 5’ and 3’ UTR of the transferrin receptor. The IRE binding protein (IRE-BP) binds to the IRE and acts as a translational repressor. In addition, binding of IRE-BPs to the IREs also controls the mRNA stability of the transferrin receptor by blocking an endonucleolytic nuclease site (Haile et al., 1989).

Once the translation initiation complex has recognized the AUG start codon, synthesis of the polypeptide chain can commence. In most eukaryotes, the protein elongation factors eEF-1 and eEF-2 are required for elongation. The rate of elongation may be controlled through phosphorylation of eEF-1 and eEF-2.

eEF-2 is phosphorylated by a specific Ca^{2+}/calmodulin-dependent eEF-2 kinase (Ryazanov and Spirin, 1990), which demonstrates that intracellular calcium not only regulates translation through phosphorylation of the initiation factor eIF2:GTP, but also through phosphorylation of the elongation factor eEF-2. Again, this mechanism is yet another example of a unexplored mechanism in receptor research.
Once the elongation complex has recognized a stop codon, translation is terminated by the two eukaryotic release factors, eRF1 and eRF3. To date it is unclear whether the rate of translation is actively modulated through regulation of eRF1 and eRF3.

2.2.11 Protein processing

Once the receptor polypeptide is synthesized it needs to be converted to its biological active form. This may involve folding the polypeptide into the correct secondary and tertiary structure, the addition of sugar residues (glycosylation), the addition of fatty acid chains, proteolytic cleavage, addition of disulfide bonds, phosphorylation and polymerization of subunits.
Further, newly synthesized protein needs to be transported to the correct location in the cell, a process that is largely determined by signal peptides and signal patches within the protein.
Although receptors may be regulated through regulation of each or these mechanisms, phosphorylation and polymerization seem to be the mechanisms that predominately control receptor function once the polypeptide has been assembled. As phosphorylation of newly synthesized proteins is almost identical to phosphorylation processes of mature proteins, this will be discussed in chapter 2.3.2.

2.2.12 Polymerization of proteins.

Receptor function may also be controlled through the interaction of different subunits.
Some receptors, like the epidermal growth factor receptor, are made of a single polypeptide chain. However, many receptors consist of more than one subunit. These polymeric receptors can be composed of either identical subunits (homomeric) or different subunits (heteromeric). For example, the ryanodine receptor is a homo-tetramere, while the muscle nicotinic acetylcholine receptor is a hetero-pentamere.
Regulation of receptor function can be mediated through the subunit composition of heteromeric receptors. For example, the embryonic muscle

acetylcholine receptor has an $\alpha 1_2 \beta 1 \gamma \delta$ composition, while the adult form has an $\alpha 1_2 \beta 1 \varepsilon \delta$ composition. In addition to its role in development, it has been demonstrated that the nicotinic receptor reverts to its embryonic form in denervated muscle tissue (Moss et al., 1987), a process that is modulated through subunit specific regulation by the MyoD and myogenin transcription factor family (Eftimie et al., 1991).

2.3 Changes in receptor properties

Once a mature and functional receptor is synthesized, it can be regulated through a number of processes. First, the receptor itself may be altered. This is usually achieved by phosphorylation (see chapter 2.3.2) and dephosporylation of the receptor (see chapter 2.3.2). Alternatively, receptors may bind regulating factors (see chapter 2.3.3). Further, the function of the receptor might be regulated through changing its location in the cell, a process known as receptor redistribution (see chapter 2.3.4). Finally, processes downstream of receptor activation may be regulated, e.g. through changes in G-protein function or changes in signal transducing cascades. Below, only the processes that directly influence the function of receptors will be discussed.

2.3.1 Phosphorylation: Protein kinases

In general, phosphorylation leads to desensitization of the receptor and is mediated through enzymes known as protein kinases. These protein kinases can be divided into two families, tyrosine-specific kinases and serine/threonine specific protein kinases.

Tyrosine-specific protein kinases are the catalytic domains of a famlily of receptors known as tyrosine kinase receptors. Examples of this family are the epidermal growth factor receptor, the platelet derived growth factor receptor and the insulin-receptor. These receptors are characterized by a protein kinase domain within the cytoplasmic region of the receptor. Upon stimulation of the receptor, the protein kinase domain phosphorylates tyrosine residues in target proteins. In addition, these receptors can phosphorylate themselves. In case of the insulin receptor, autophosphorylation stimulates the tyrosine kinase activity of the receptor (Kasuga et al., 1982; Herrera and Rosen, 1986).

The aminoacid residues serine and threonine in a receptor can be phosphorylated by serine/threonine-specific protein kinases. This mechanism of phosphorylation has proven to be important for the regulation of the large family of seven transmembrane spanning receptors. Unlike the tyrosine-specific kinases, the serine/threonine-specific protein kinases are not part of the receptor. Examples of serine/threonine-specific protein kinases are protein kinase C (PKC), protein kinase A (PKA), calmodulin-dependent protein kinases and G-protein coupled receptor kinases, although many others exist.

PKC is activated through stimulation of the inositol phospholipid pathway and may lead to the direct phosphorylation of receptors. For example, phosphorylation by PKC and subsequent desensitization of the seven transmembrane spanning receptor for histamine (Fujimoto et al., 1999) has

been reported recently. In addition, PKC can also desensitize tyrosine-kinase receptors, like the insulin receptor, through phosphorylation of a serine residue (Bollag et al., 1986). Further, PKC mediated phosphorylation has been reported for the neuronal inositol 1,4,5-trisphosphate receptor (Nucifora et al., 1995) where alternative splicing generated an extra PKC phosphorylation site.
PKA is activated by increased levels of the second messenger cAMP. Like PKC, PKA mediated phosphorylation of numerous receptors families have been described, including tyrosine-kinase receptors (Barbier et al., 1999) and seven transmembrane spanning receptors (Paradiso and Brehm, 1998).
The family of calmodulin-dependant protein kinases, of which Ca-kinase II is the most prominent, is thought to mediate most of the phosphorylation-related actions of intracellular Ca^{2+} signaling. An integral part of these protein kinases is the small molecule calmodulin. After activation by increased intracellular free calcium, calmodulin binds and activates the calmodulin-dependant protein kinases. For example, the function of the epidermal growth factor receptor can be regulated through phosphorylation by Ca-kinase II (Feinmesser et al., 1999). In addition to binding to protein kinases, calmodulin can influence receptor function by binding directly to receptors (see chapter 2.3.2).
The last example of protein kinases that control receptor function is the family of G-protein coupled receptor kinases. Once a seven transmembrane spanning receptors is in its active state, it may be recognized and desensitized by receptor specific G-protein coupled receptor kinases. For example, the α_{1B}-adrenergic receptor can be desensitized both by a G-protein coupled receptor kinases and by PKC (Diviani et al., 1997). Further, phosphorylation of G-proteins by specific G-protein coupled receptor kinases facilitates the binding of negative regulatory proteins known as arrestins (see chapter 2.3.3)
As mentioned earlier, in addition to regulating receptors through the direct phosphorylation of receptors, protein kinases may also regulate the transcription factors that control the transcription of receptor genes, e.g. the transcription factors CREB (Andrisani, 1999) and MyoD (Eftimie et al., 1991).

2.3.2 Dephosphorylation: Protein phosphatases

Phosphorylation of receptors by protein kinases is a process that can be reversed by a group of enzymes called protein phosphatases. These protein phosphatases are characterized by the aminoacid residues that they can dephosphorylate. First, the protein tyrosine phosphatases can dephosphorylate tyrosine residues of several proteins, including tyrosine kinase receptors. The second class of protein phosphatases can dephosphorylate serine and threonine

residues and are therefore called the serine/threonine protein phosphatases (for review see (Wera and Hemmings, 1995)). Finally, some protein phosphatases are able to dephosphorylate both tyrosine and serine/ threonine residues and are called the dual-specificity protein phosphatases.

At present, some 50 mammalian protein tyrosine phosphatases (PTPs) have been described. However, the total number of PTPs in the human genome has been estimated at 500 (Tonks and Neel, 1996). One of the first PTPs described is CD45, a transmembrane PTP that is expressed on the surface of all nucleated hematopoietic cells. In addition to transmembrane PTPs, some PTPs are present in the cytosol or in the endoplasmic reticulum.

The PTPs can be divided into two groups, the "classical" PTPs and the low molecular weight PTPs. The "classical" PTPs are characterized by one or more conserved ~250 amino-acid catalytic domains and represents the largest family of PTPs. An example of "classical" PTP mediated receptor regulation is given by the dephosphorylation of the insulin receptor by PTP1B (Seely et al., 1996).

The low molecular weight PTPs (LMPTPs) are characterized by their low molecular weight and are highly conserved in evolution (Ramponi and Stefani, 1997). Little is known about the physiological role of LMPTPs, although LMPTP mediated modulation of the platelet-derived growth factor receptor (Berti et al., 1994) and insulin receptor (Chiarugi et al., 1997) has been described.

Based on their biochemical properties, the serine/ threonine phosphatases can be divided into two classes, type-1 protein phosphatases (PP1) and type-2 protein phosphatase (PP2). PP1 can be inhibited by two heat stable inhibitors, called inhibitor-1 and inhibitor 2, whereas PP2 is insensitive to these inhibitors. The class PP2 can be subdivided into spontaneously active (PP2A), Ca^{2+}-dependent (PP2B) and Mg^{2+}-dependent (PP2C) classes.

PP1 is involved in many cellular processes and PP1 mediated receptor dephosphorylation has been demonstrated for a number of receptors, including the ryanodine receptor (Hain et al., 1994; Zhao et al., 1998) and the glutamate receptor (Snyder et al., 1998). PP1 exists in several isoforms, which are regulated by different mechanisms. The glycogen-bound PP1 from skeletal muscle is primarily regulated through phosphorylation by PKA, binding of the type 1 inhibitor DARPP-32, binding of inhibitor 2 and by dephosphorylation by PP2A, PP2B and PP2C. Glycogen-bound PP1 from liver is primarily regulated through phosphorylase *a*. Finally, the nuclear isoform of PP1 can be activated by PKA and casein kinase II mediated phosphorylation of its inhibitor NIPP-1.

The involvement of the spontaneously active PP2A in regulating receptor function, has been demonstrated for a number of receptors, including the ryanodine receptor (Herrmann-Frank and Varsanyi, 1993), the guanylyl cyclase-A receptor (Potter and Garbers, 1992), the transferrin receptor (Runnegar et al., 1997) and opioid receptor (Muranyi et al., 1997). PP2A can be regulated through phosphorylation by tyrosine kinases, the epidermal growth factor and insulin receptor. In addition, PP2A is regulated by methylation of a C-terminal sequence by a carboxymethyltransferase. Besides directly dephosphorylating receptors, PP2A may also regulate receptor levels through regulation of transcription factors like CREB (Muranyi et al., 1997).
The Ca^{2+}-dependent PP2B is also known as calcineurin. PP2B can bind to calmodulin and has structure similar to calmodulin. PP2B is regulated by intracellular Ca^{2+} levels and is active at lower Ca^{2+} concentrations than the calmodulin-dependant protein kinases. The involvement of PP2B in regulating receptors has been demonstrated for several receptors, including the type-1 inositol 1,4,5-trisphosphate receptor (Genazzani et al., 1999; Cameron et al., 1997; Cameron et al., 1995a), the 5-HT3 receptor (Boddeke et al., 1996) and the $P2_{X3}$ receptor (King et al., 1997).
The protein phosphatase PP2C has been characterized as a Mg^{2+} dependent enzyme. However, the involvement of PP2C in regulating receptors has not yet been described.
Examples of dual-specificity protein phosphatases are the human VHR and the MAP kinase phosphatases. Although the human VHR is expressed in most tissues, little is known about its physiological role. However, dephosphorylation of both tyrosine and serine residues by VTR has been observed for the keratinocyte growth factor receptor (Ishibashi et al., 1992).
The MAP kinase phosphatases are a class of protein phosphatases that are specific for MAP kinases. MAP kinases are serine/ threonine protein kinases that are activated as an early response to stimuli involved in cell growth and differentiation. The involvement of MAP kinases/ MAP kinase phosphatases in receptor regulation is primarily at the level of transcriptional control, as is demonstrated for the transcriptional activation of acetylcholine receptor subunits (Si and Mei, 1999)

2.3.3 Receptor-binding factors.

Receptors may also be regulated by binding regulatory factors. For example, it has been demonstrated recently that binding of calmodulin to the type I inositol 1,4,5-trisphosphate receptor inhibits the binding of inositol 1,4,5-trisphoshate

to its receptor (Sipma et al., 1999). Calmodulin binding sites have also been identified in a distant relative of the inositol 1,4,5-trisphosphate receptor family, the ryanodine receptor (Menegazzi et al., 1994). As both the inositol 1,4,5-trisphosphate receptor and the ryanodine receptor are calcium release channels, calmodulin provides feedback regulation of Ca^{2+} on receptor function.

Another example of a regulatory protein that can bind to receptors is the family of FK506-binding proteins (FK506BP). FK506BP are the intracellular targets for FK506, an immunosuppressive drug that prevents T-cell activation. FK506BP is widely expressed in eukaryotic cells and can bind to several receptors including the ryanodine (Qi et al., 1998) and inositol 1,4,5-trisphosphate receptor (Cameron et al., 1995b). FK506BP presumably modulate receptor function by stabilizing its closed or inactive conformation (Mayrleitner et al., 1994). In addition, it has been shown that FK506BP facilitate the binding of calcineurin (also known as PP2B, see chapter 2.3.2) to the inositol 1,4,5-receptor, resulting in receptor phosphorylation and a modulated Ca^{2+} response.

The last example of regulatory proteins is the family of arrestins. After receptor activation, many G-protein coupled receptor receptors (GPCR) are rapidly phosphorylated by G-protein coupled receptor kinases (see chapter 2.3.1). Phosphorylation dramatically increases the affinity of the receptor for arrestins, as has been demonstrated for the β_2-adrenergic receptor (Lohse et al., 1992; Gurevich et al., 1995). To date, four mammalian arrestins have been described; visual arrestin and cone arrestin are restricted to the phototransduction pathway, whereas β-arrestin and β-arrestin-2 are ubiquitously expressed.

Arrestins uncouple the receptor from its G-protein by binding to its intracellular loops, thus interfering with the signaling between the GPCR and the G-protein (Gurevich et al., 1994). The uncoupling of the receptor results in desensitization of the receptor. In addition to uncoupling of GPCRs, arrestins may also serve as adapters for the binding of clathrin (Goodman et al., 1996), a component important for endocytosis of GPCRs (see chapter 2.4.2).

In addition to the regulation by regulatory proteins, receptor function may also be influenced by binding smaller molecules like ATP and inositol 1,3,4,5-tetrakisphosphate (InsP4) or even by ions like Ca^{2+}.

For example, ATP and Ca^{2+} can bind to the inositol 1,4,5-trisphosphate receptor and stimulate this receptor at low concentrations of ATP and Ca^{2+}, whereas at high concentrations it inhibits the receptor (Missiaen et al., 1997; Missiaen et al., 1997).

2.3.4 Receptor redistribution

The location of receptors within a cell is to a large extent controlled by signal peptides and signal patches within the protein. However, the position of a receptor may change over time. The movement of a receptor to a new position in the cell in a process called translocation may enable the cell to adapt to changing conditions. Alternatively, the receptors may be moved from their original positions to degrading organelles, a process that will be discussed in chapter 2.4.

Translocation from the cytosol into the nucleus is an important regulatory mechanism for steroidreceptors. For example, the glucocorticoid receptor resides primarily in the cytoplasm in its inactivated form. After binding a steroid, the glucocorticoid receptor undergoes a conformational change and translocates to the nucleus, where it influences gene transcription.

In order to enter or exit the nucleus, receptors need to pass the nuclear pore complex (NPC), a complex protein structure spanning the inner and outer nuclear membrane. Although small molecules may pass the NPC passively by diffusion, larger molecules require energy-dependent processes to enter the nucleus. Therefore, regulation of nuclear-translocating receptors may be controlled by the mechanisms that control the active transport of proteins across the NPC. However these mechanisms are still not well investigated.

2.4 Proteolytic degradation/ recycling of receptors

The final mechanism in receptor regulation is the regulation of receptor degradation by proteolytic pathways. Three major pathways have been identified in the degradation of proteins and receptors. First, lysosomal degradation is an important proteolytic pathway for degradation of plasmamembrane receptors. Because lysosomes are intracellular organelles, receptor internalization is required for the degradation of plasmamembrane receptors by this pathway. Secondly, the ubiquitin/ proteosome pathway represents an important proteolytic pathway for the degradation of both cytosolic and membrane bound receptors. Finally, the calpain proteolytic pathway is important for the patrial degradation of membrane associated receptors.

2.4.1 Endocytosis of receptors: degradation or recycling.

Activation of many receptors results in endocytosis, a process also known as internalization. In this process, small patches of plasmamembrane bud from the plasmamembrane to form small intracellular vesicles. These vesicles may contain membrane-associated proteins, including plasmamembrane receptors.

Several receptors, like the EGF receptor, are quickly aggregated into clathrin-coated pits upon ligand binding (Lamaze et al., 1993). Other receptors are clustered in the clathrin-coated pits and internalized regardless of receptor ligand binding. Examples of such receptors are the transferrin and mannose-6-phosphate receptor.

The best characterized endocytic pathway is through clathrin-coated pits, although non-coated vesicle pathways have been described (for review see (Lamaze and Schmid, 1995)).

Endocytosis through the clathrin-coated vesicle pathway is mediated through specialized regions of the plasma membrane that contain lattices of clathrin. Clathrin has a trimeric structure, which enables the formation of both flat and rounded lattices. Electron micrographs revealed that the eukaryotic plasmamembrane contains areas of both flat clathrin lattices as well as invaginated rounded lattices, known as clathrin-coated pits.

Clathrin can self-assemble into cage-like structures. However, under physiological conditions, the presence of adapter molecules facilitates the formation of the cage-like structure. In addition, these adapter proteins mediate the binding of clathrin to the plasmamembrane and to plasmamembrane receptors. Recent studies suggest that arrestins may also function as adapter proteins (Goodman et al., 1996).

Endocytosis mediated through clathrin coated pits, is achieved through the invagination of the clathrin-coated pits and the subsequent budding into intracellular vesicles. Although the regulation of this process is still poorly understood, several regulatory molecules have been identified. For example, dynamin is thought to act as a collar around deeply invaginated coated pits, which then closes to form free clathrin-coated vesicles. In addition, phospholipase D seems to regulate the binding of adapter protein 2 (AP-2) to the plasmamembrane and to endosomal compartments (West et al., 1997). Further, the adapter function of both AP-2 and arrestins is controlled by their phosphorylation status (Wilde and Brodsky, 1996; Lin et al., 1997) as well as by binding phosphoinositides (Timerman et al., 1992; Gaidarov et al., 1999)

Upon formation, the endocytic vesicles fuse with intracellular organelles known as endosomes. The endocyted receptors are then processed through several endosomal compartments known as early, intermediate and late endosomes. In a process known as endosomal sorting, the proteins in the endosomes are sorted into a fraction that is to be recycled to the plasmamembrane and a fraction that is to be degraded in the lysosome. Although the mechanisms of endosomal sorting is still largely obscure, it has been demonstrated that the fate of receptors in these endosomes depends on specific signals within the cytoplasmic tail of receptors (White et al., 1998) and receptor-occupancy (Herbst et al., 1994).

Endosomal sorting provides an elegant way of receptor down-regulation as has been demonstrated for the EGF receptor. The EGF receptor is constantly internalized at a stable rate, but is also rapidly recycled. However, occupied EGF receptors are recycled at a much lower rate due to retention within recycling endosomes (Herbst et al., 1994), resulting in down-regulation of EGF receptors.

The last step in endocytosis is the fusion of the late endosomes with lysosomes, resulting in the degradation of the endosomal content.

2.4.2 lysosomal degradation of receptors.

Lysosomes are specialized organelles that contain a mixture of proteases, peptidases and hydrolases. The lysosomal proteases that are responsible for the degradation of receptors and other proteins are collectively known as cathepsins.

In order to be degraded, receptors need to be transported to the lysosomes, which can be achieved through several mechanisms, including endocytosis (see

chapter 2.4.1), autophagy and direct protein transport through the lysosomal membrane.
Autophagy is the process in which a region of the cytoplasm is surrounded by a membrane. The content of newly formed compartment is subsequently degraded after fusion with a lysosome. In the liver, autophagy is one of the main pathways for the degradation of intracellular macromolecules and organelles. Although intracellular receptors may be affected by autophagy, specific regulation of receptors by autophagy has not been described.
Finally, proteins may be transported to the lysosome through direct protein transport mechanisms. Substrate proteins for direct transport to the lysosome are recognized by the aminoacid sequence KFERQ (Dice and Chiang, 1989). Binding of the cytosolic chaperone hsc73 to the KFERQ motif directs the complex towards the lysosome. There, the complex is recognized by a receptor (Cuervo and Dice, 1996), internalized and degraded. Although the KFERQ sequence is present in about 30% of all intracellular proteins (Dice and Chiang, 1989), the involvement of the direct protein transport pathway in receptor regulation has not yet been described.

2.4.3 Proteosomal degradation: protein stability
In addition to the KFERQ sequence, the stability of proteins is also determined by the first aminoacid residue on the aminoterminal side. The aminoresidues Met, Ser, Thr, Ala, Val, Cys, Gly and Pro are stabilizing residues, while the remaining twelve aminoacids enhance degradation. As all newly synthesized proteins are generated with an initial stabilizing Met residue, these proteins needs to be processed in order to obtain a different aminoterminal residue. The Met residue can be removed by specific aminopeptidases. Alternatively, a destabilizing residue can be added to the aminoterminal residue by the enzyme aminoacyl-tRNA protein transferase.
To date, it is unknown whether receptors are regulated by changing the first aminoacid residue

2.4.4 Proteosomal degradation: ubiquitin dependent protein degradation.
One of the best studied cytosolic proteolytic pathways is the ubiquitin dependent protein degradation by the 26S proteosome. The ubiquitin pathway involves two distinct and successive steps. First, proteins to be degraded are tagged by the convalent binding of 8.6 kDa proteins called ubiquitin molecules. Secondly, the ubiquitin tagged proteins are degraded by either proteosomes or in some cases in lysosomes.

The "tagging" of proteins with ubiquitin molecules, is performed by a complex machinery of enzymes that can be divided into three groups. The first group of enzymes are the ubiquitin activating enzymes that activate ubiquitin in its C-terminal Gly. The second group consists of ubiquitin transfer enzymes that transfer activated ubiquitin to the third group of enzymes, the ubiquitin conjugating enzymes. The ubiquitin conjugating enzymes have diverse characteristics and "tagging' of specific proteins is achieved through the use of conjugating enzymes that are specific for certain substrates.
Recognition sites for ubiquitin-conjugating enzymes may be the first N-terminal aminoacid residue that controls protein stability (see chapter 2.4.4). Alternatively, the ubiquitin conjugating enzymes may recognize modified proteins (for example phosphorylated proteins) or ancillary proteins. Ubiquitin may also be attached to itself, forming a multiubiquitin chain on a substrate protein.
The effect of the ubiquitin conjugating enzymes can be reversed by enzymes that remove the ubiquitin groups, enabling the proteins to be recycled instead of degraded.
Once a substrate molecule is polyubiquitinated it can be degraded by the 26S proteosome, a complex of the 20S proteosome and several regulatory proteins. Protein degradation by the 26S proteosome is dependent on ATP, however the exact mechanisms involved are not well understood yet.
Although ubiquitin dependent protein degradation by the 26S proteosome is important for the degradation of short-lived and abnormal proteins, several studies have shown that receptor degradation is also mediated by this pathway. For example, the involvement of the ubiquitin/ proteosome pathway in the degradation of receptor proteins has been demonstrated for the inositol 1,4,5-trisphosphate receptor (Bokkala and Joseph, 1997; Oberdorf et al., 1999), the progesterone receptor (Syvala et al., 1998), the EGF receptor (Mori et al., 1997; Galcheva-Gargova et al., 1995), the growth hormone receptor (Strous et al., 1996), the PDGF receptor (Mori et al., 1995) and the Met tyrosine kinase receptor (Jeffers et al., 1997).

2.4.5 Calpain mediated protein degradation.

The second major cytosolic proteolytic pathway is dependent on calpains or calcium-dependant proteases. Calpains are involved in the partial degradation of membrane and cytoskeletal proteins.
Calpains in the cytosol are usually bound to an inhibitor molecule called calpastatin, which inactivates the calpains. Upon an increase of intracellular

calcium, calpain is dissociated from calpastatin and translocated to a cellular membrane where it is activated by autolysis.

Receptor degradation by calpains has been demonstrated for a number of receptors including the inositol 1,4,5-trisphosphate receptor (Sipma et al., 1998), the NMDA receptor (Bi et al., 1998), the common cytokine receptor (Noguchi et al., 1997), the retinoid X receptor (Matsushima-Nishiwaki et al., 1996), the glutamate receptor (Bi et al., 1996), the EGF receptor (Gregoriou et al., 1994), and the insulin receptor (Smith et al., 1993). Calpain degradation of inositol 1,4,5-trisphosphate receptor may provide a negative feedback loop of inositol 1,4,5-trisphosphate receptor mediated Ca^{2+} release on inositol 1,4,5-trisphosphate receptor levels.

2.5 Future directions:

In this chapter, an overview has been given on the mechanisms that regulate receptor synthesis, receptor properties and receptor degradation. From this it can be concluded that receptors can be regulated through many different mechanisms. Although some mechanisms have received a lot of attention this does not necessarily mean that these mechanisms are well-understood. E.g. numerous articles have reported on intron-exon splice sites in receptor genes, but the mechanisms that control splicing are still not well-understood.

Studies on receptor synthesis have focused primarily on transcription, either by studying mRNA levels or promoter activity. Historically, the transcriptional rate of a receptor gene was determined by measuring receptor mRNA levels using techniques like RNA protection assays, competitive PCR and Northern blotting. With the cloning of promoter sequences, transcription may now also be studied using promoter-reporter assays (see 2.2.3).

Several mechanisms that may control receptor synthesis have been poorly investigated. E.g. the mechanisms that control the export of the mRNA out of the nucleus are still poorly understood. Further, specific regulation of receptor mRNA's by translational mechanisms may deserve more attention.

Receptor regulation through changing receptor properties has primarily focused on phosphorylation/ dephosphorylation mechanisms and on regulatory factors. However, translocation or redistribution of receptors within the cell is still a relatively unexplored field. Recent research indicated that signaling events at the cell surface are compartmentalized and integrated by caveolae. These caveolae are specialized receptor-rich plasmalemmal microdomains that are distinct from clathrin coated pits. Regulation of receptor translocation to and from these caveolae may prove to be an important mechanism in receptor regulation.

Proteolytic degradation of receptors has received considerable scientific attention. Although many studies have reported on receptor regulation through enhanced degradation, the underlying processes are still poorly understood. E.g. endocytosis through clathrin-coated pits has been studied extensively but endocytosis through non-coated vesicle pathways (i.e. through caveolae) have not been elucidated. Further, the processes that control receptor degradation and recycling are still largely unknown.

In conclusion, receptors can be regulated through many different mechanisms. Some of these mechanisms have remained relatively unexplored and may therefore provide new insights into receptor regulation and receptor intervention.

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